PROTOCOL FOR EVALUATION OF THE RESPONSE OF METASTATIC

LOX AMELANOTIC MELANOMA TO CHEMOTHERAPEUTIC AGENTS

MODEL: (3L032) Treatment of established subcutaneous tumors followed by bioassay of lung tissue with metastases.

Origin of Tumor Line: This line was obtained from Dr. Oystein Fodstad, Department of Biochemistry, Norwegian Radium Hospital, Oslo, Norway.

Summary of Test Procedures: lO(6) LOX ascites cells are implanted subcutaneously (SC) near the axillary region of athymic random bred (NCr-nu) mice. The sc tumors are allowed to grow from 15 to 25 days. Various treatment schedules may be used, but the final treatment should be between Days 15 and 25 postimplant. Twenty-four hours after the last treatment, the animals are sacrificed and their lungs removed. The lungs are then implanted sc into other animals to assay for tumor growth (this is called a bioassay). The animals that were treated and sacrificed for lung removal will be referred to as "donors". The bioassay animals in which the lungs were implanted sc will be referred to as "recipients." The parameter used will be "cell kill" and will be based on the delay in tumor appearance from the treated tissue as compared to tumor appearance from untreated control tissue in the recipients.

ANIMALS: (refer to Protocol 8)

Propagation:	Athymic NCr-nu
Testing:	Athymic NCr-nu
Weight:	Minimum weight of 18 gm for males and 17 gm for females.
Sex :	One sex is used for all tests, titrations, and control animals in one experiment.
Source:	One source for all animals in one experiment. Exceptions must be noted as comments.

EXPERIMENT SIZE:

Donor Animals

Treatment Groups: 6 animals

Control or Dose 0 Group: 10 animals

Recipient Animals (Bioassay)

From Treatment Groups: Bioassay into 6 animals

From Control or Dose 0 Group: Bioassay into 10 animals

TUMOR TRANSFER: (refer to Protocols 2, 5, and 6)

Propagation:

Tissue:

Suspension: Prepare diluted ascitic fluid containing 1 x lO(7) cells (approximately 0.1 ml). (Refer to the following section entitled Preparation of Cell Suspension).

Time: Days 7-11 (Harvest should be done between the time the animals become obviously symptomatic and before the ascitic fluid becomes dark red.)

Site: Implant 1 x 10(7) cells/animal injecting approximately 0.1 ml per mouse ip using a 0.5 inch 23 gauge needle with a 1 ml tuberculin syringe. Site of injection should be chosen to avoid vital organs.

Testing:

Tissue:

Suspension: Prepare 0.1 ml of diluted ascitic fluid containing 1 x lO(6) cells. (Refer to the following section entitled Preparation of Cell Suspension.)

Time: Days 7-11 (Harvest should be done between the time the animal becomes obviously symptomatic and before the ascitic fluid becomes dark red.)

Site: Implant 1 x lO(6) cells/animal injecting 0.1 ml per mouse S.C. using a 0.5 inch,

23 gauge needle with a 1 ml tuberculin syringe. Site of injection should be chosen to avoid vital organs.

PREPARATION OF CELL SUSPENSION:

Use donor tumor when the abdomen becomes distended (usually Days 7 through 11) · Using a sterile 5 ml syringe, withdraw ascitic fluid asceptically through the abdominal wall from which the skin has been removed. Use a minimum of 3 mice. Collect at least 3 ml of ascitic fluid. Pool fluid in a sterile glass container held in an ice bath.

Cell Count: Use physiological saline for dilutions. Use serological pipettes with rubber bulb attachment. Cell suspensions should be swirled and mixed by aspirating the solution into and out of the pipette several times before withdrawing an aliquot.

Dilutions:

Suspension A: 0.5 ml of pooled ascitic fluid plus 4.5 ml of physiological saline.

Suspension B: 0.5 ml of Suspension A plus 4.5 ml of physiological saline.

Suspension C: 1.0 ml of Suspension B plus 4.0 ml of physiological saline. Suspension C is a 1:500 dilution and should be used to make the cell count as follows:

1. Agitate Suspension C and withdraw 0.5 ml with a l-cc syringe and a 23-gauge needle.
2. Agitate and allow several drops to flow out, then fill both chambers of the hemacytometer.
3. Count only intact nucleated cells using 100x to 400x magnification.
4. Assuming the use of an A0 STD or comparable hemacytometer, count 4 large squares in both chambers, being sure to establish and follow a convention for inclusion of cells that fall on lines.

The cell count may be calculated directly from the number of cells in both hemacytometer chambers as follows:

$$\frac{\text{Total No. of Cells In Both Hemacytometer Chambers}}{2} \times 2.5 \times 500 \times 1000^{*} = \text{Cells/ml of the Undiluted Ascitic Fluid}$$

Note: Once the cell count has been determined, there is no further need for Suspensions A, B, or C. The cell suspension for inoculation will be made from the original undiluted ascites from which Suspension A was made.

The dilution factors required to prepare a suspension of cells that contains the highest cell number required in 0.1 ml may be calculated as shown in the following example:

$$\frac{\text{10,000,000 (Cells/ml Required for 1 x 10(6) in 0.1 ml Implant)}^{**}}{\text{85,000,000 (Cell Count/ml of Ascitic Fluid)}} = \frac{X \text{ ml (ascites)}}{\text{10 ml (total volume)}}$$

$85X = 100$

$X = 1.18$ ml

Add 1.18 ml of ascitic fluid to 8.82 ml of physiological saline for a suspension of 1 x 10(6)cells in 0.1 ml.

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*2.5: Correction factor to convert count to cells per cubic mm.

500: Dilution factor.

1000 : Converts mm (3) to ml.

**For stock passage and titration, this value should be 100,000,000 (cells/ml required for 10(7) in approximately 0.1 ml implant)

Inoculation: Sterile procedures should be followed to dilute the ascitic fluid to obtain Suspension 1 and subsequent dilutions.

Suspension 1: Highest level required; 1 x lO(6) cells in 0.1 ml.

Suspension 2: Add 1 part of Suspension 1 to 9 parts of physiological saline = 1 x lO(5) cells in 0.1 ml.

Suspension 3: Add 1 part of Suspension 2 to 9 parts of physiological saline = 1 x 10(4)cells in 0.1 ml.

Suspension 4: Add 1 part of Suspension 3 to 9 parts of physiological saline = 1 x lO(3)cells in 0.1 ml.

Use sufficient numbers of sterile syringes and needles so that no syringe will be refilled from the pool of donor fluid. No more than 60 minutes should elapse from the time fluid is taken from the donor until it is implanted in all of the recipient animals. For titrations, inoculate the lowest level first, then proceed to inoculate each higher level.

Note: Although the LOX amelanotic melanoma is passaged from ascites-free cells, there will be some solid tumor formation in the peritoneal cavity. This solid tumor formation becomes more pronounced with time after implant. Also, this tumor appears to produce a mucus-like condition that results in thick ascitic fluid. It may be necessary to inject 1 or 2 ml of physiological saline in order to facilitate harvest.

Testing Schedule:

Variable: The metastatic burden will be relatively small on Day 15 and well established on Day 25 postimplant. The last day of treatment should fall between Days 15 and 25.

Dav 0: Implant tumor (lO(6) cells sc). Run bacterial cultures (refer to Protocol 7 and Instruction 348). Prepare materials. Refer to Protocol 10 or Instruction 361 for instructions on randomization. Record deaths daily.

Dav 1: Check Cultures. Discard experiment if contaminated. Weigh animals individually and record weights. Treat as instructed.

Day 2: Recheck cultures. Discard experiment if contaminated.

Treatment Day(s) Administer test agent based on individual body weight.

Measurement and Weigh Davs: The first day of treatment and bioassay day.

Positive Control: NSC 26271 (cyclophosphamide), 200 mg/kg/dose, single dose, 24 hours prior to bioassay. The delay in tumor appearance (T-C) is not established, but should be >7.0 days.

Bioassav Dav: Sacrifice each animal, remove the lungs and divide into two parts. Mince one part into small pieces and place in a 3 cc Luer Lok syringe (with plunger removed), add 1 cc of saline, replace the syringe Plunger and put on an 18-gauge needle. Work the excess air out of the syringe.Insert the needle sc near the axillary region of a recipient animal on the right side. Force the tissue through the needle caution: use safety glasses or face cover). Repeat this process using the other lung portion to make an implant on the opposite side of the same animal. Since some time will be required for this procedure, sacrifice only one group at a time.

Measurement Davs: Palpate and measure tumors on the recipient animals twice weekly. (Because of the large amount of lung tissue implanted, a tumor must obtain a size of 500 mg before one can be sure it is a tumor.)

Evaluation: Based on the difference in the day of tumor appearance to 500 mg from treated and untreated metastases (T-C). Cell kill may be estimated as follows:

$$\text{Cell Kill (log units)} = \frac{0.3\ (T-C)}{td}$$

Where: 0.3 = the log of 2.0
td = the tumor doubling time.

T = Median day of appearance of the treated tumors to a size of 500 mg

C = Median day of appearance of the control tumors to a size of 500 mg

Note: The td can be estimated by plotting the growth of the control tumors.

Criteria for Activity: Not established, although 1.0 log $_{10}$ unit of cell kill would suggest at least minimal activity.

Reporting of Data: Special Report.